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PRODUCTION OF SOLUBLE HUMAN CLASS I PROTEINS FROM cDNA

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation of U.S. Serial No. 09/465,321 filed December 17, 1999, now abandoned.

STATEMENT REGARDING FEDERALLY FUNDED RESEARCH

[0002] At least a portion of the invention was developed under funding from the National Institute of Health ("NIH") under contract Nos. No1-A1-45243 and No1-A1-95360. As such, the Government may own certain rights in and to this application.

BACKGROUND OF THE INVENTION

[0003] Immunology may be defined as the study of the body's reaction to foreign bodies, including microbes as well as macromolecules such as proteins and polysaccarides, without specifying the physiological or pathological consequences of such reactions.

[0004] In the last few years recognition and definition of the multitude of genes in the human body have been proceeding at a rapid rate, and major advances in the understanding of disease treatment and prevention is

occurring.

[0005] The human body has a family of highly polymorphic genes called the major histocompatiblity complex (MHC) which encode transmembrane proteins. Class I and class II MHC molecules play a central role in most if not all adaptive immune responses because class I and class II MHC molecules present antigens to T lymphocytes. The human class I and class II MHC molecules are known as HLA (Human Leukocyte Antigen) molecules.

[0006] Aside from their central role in directing immune responses against pathogens, the MHC class I and class II molecules are distinguished by their extensive polymorphism. This extensive polymorphism which leads most individuals to be immunologically different, is what causes tissue or transplant rejection between individuals. Differences in the MHC from one individual to another are also why some individuals are susceptible to infections while others are not. Particular MHC types are associated with autoimmune disorders such as diabetes and arthritis.

[0007] Class I and class II HLA molecules contribute significantly to autoimmunity, transplantation, susceptibility and/or resistance to infectious disease, and cancer. For these reasons a detailed understanding of the biological role of MHC proteins in immune responses is now being sought. To realize the role of HLA class I and class II molecules in human disease requires HLA class I and class II proteins. Clinical therapies to manipulate or alter

immune agents which interact with HLA class I and class II molecules will also require MHC proteins.

[0008] An example of the research tests in which MHC class I and class II molecules are used include:

- MHC-peptide multimers used as immunodiagnostic reagents for disease resistance/autoimmunity
- Assessing the binding of potentially therapuetic peptides
- Elution of peptides from MHC molecules to identify vaccine candidates
- Screening transplant patients for preformed MHC specific antibodies
- Removal of antibodies from a patient

[0009] Because these studies are based around human diseases, MHC molecules produced in humans will most readily lead to accurate research results in humans. MHC molecules produced in humans will also lead to immune based intervention and therapy in the clinic.

[0010] There are several research and clinical tests that MHC molecules can be and are used in. These include clinical crossmatch tests for solid organ and bone marrow transplantation (FACS, ELISA, columns), peptide binding tests which examine the ability of potentially therapuetic peptides to bind to various MHC class I and class II molecules (reference the work of Sette, Buus, Takiguichi, Ramnennsee), and tests for assessing the nature of immune

responder cells which provide disease resistance and which drive autoimmune responses (reference Altman, McMichael).

[0011] At this point in time there is no readily available source of HLA class I or class II molecules. For research tests which require relatively pure class I or class [lithe] II MHC product can be made in bacterial cells. Such is the case for class I molecules used in research experiments. Although the HLA molecules produced in bacteria will not be glycosylated or have human peptides loaded into them, bacterial production is the only means by which enough class I can be produced in a pure form for experiments. Once produced in bacteria the researchers must then load peptide(s) and light chain onto the bacterial class I heavy chain. Only then can experiments be performed.

[0012] When HLA molecules are required from human cells, laboratories typically grow up large volumes (50-100 L) of static cultures (of cells expressing multiple surface-bound HLA class I molecules) in roller bottles, following which they pellet the cells and perform established immunoprecipitation protocols to recover the multiple HLA class I molecules from cell lysates. Using these procedures, the product obtained is typically in the amount of ~300-500 µg. Note that the product obtained in this manner represents a mixture of the six different HLA class I molecules expressed by most cell lines. Interpretation of results can therefore be difficult because one cannot be certain that any particular HLA molecule is responsible for a given result. Only indirect

conclusions can be reached from these mixtures.

[0013] The present invention comprises equipment and processes for producing relatively large volumes of pure Class 1 molecules at a reasonable cost.

[0014] Class I human leukocyte antigens (ELLA), which are expressed by and present upon virtually every nucleated cell in the body, bind and display cytoplasmically-derived peptide antigens on the cell surface. The peptides they present are derived from either normal endogenous proteins ('self') or foreign proteins present within the cell ('nonself'); foreign proteins include products of malignant transformation or intracellular pathogens such as viruses. Class I molecules thus convey information regarding the internal fitness of a cell to CD8+ cytotoxic T-lymphocytes (CTLs), which are activated by interaction with 'nonself' peptides and lyse or kill the cell presenting them. Lymphocytes are a type of white blood cell or leukocyte that circulate in the lymph.

[0015] HLA class I molecules exhibit extensive polymorphism, which is generated by systematic recombinatorial and gene conversion events; it is for this reason that hundreds of different HLA types exist throughout the world's populations. Most people therefore differ in their MHC class I molecules.

[0016] Transplantation between individuals with different class I and class II MHC molecules leads to the production of strong immune responses. Some of these immune responses can be controlled with drugs. The immune

responses that can be controlled with drugs are new immune responses, or immune responses that form after the transplant. In fact, immunosuppresive drugs are now so effective that it is becoming much more common to transplant organs that are not well matched for their MHC class I and class II molecules. However, immunosuppresive drugs cannot stop the rejection of an organ transplant when the organ recipient has circulating antibodies which recognize the organ being transplanted. A number of events can trigger the production of antibodies against MHC class I molecules in other individuals. These events include blood transfusion, pregnancy, bacterial infections, and other less understood events. So, it is not uncommon for an individual who needs a heart, kidney, lung, or liver transplant to have circulating antibodies which would immediately attack some transplanted organs. These circulating antibodies cannot be inhibited with drugs, and transplanting an organ that is recognized by such antibodies will lead to organ failure before the transplant operation is finished.

[0019] The limiting reagents in determining if a patient is producing circulating antibodies that would attack a transplanted organ are Class I and Class II pure proteins. There is no good source of Class I and Class II to screen a patient's antibodies against at present. The present invention is aimed at filling this need for Class I MHC molecules. Because complete organ matching is becoming less common, the method of the present invention, including the

ability to screen for preexisting antibodies against the Class I HLAs on organs to be transplanted, is very important.

SUMMARY OF INVENTION

[0020] The present invention encompasses a method for production of individual Class I major histocomptability complex (MHC) molecules which are secreted from mammalian cells in a bioreactor unit.

[0021] Class I MHC molecules are ordinarily expressed on the cell in a membrane-bound form; they consist of an extracellular domain, a transmembrane domain, and a short cytoplasmic domain. In one embodiment of the method of the present invention, the DNA encoding the Class I MHC molecules is modified using PCR so that the Class I MHC molecules expressed from the DNA do not have transmembrane or cytoplasmic domains; since the Class I MHC proteins are no longer anchored in the membranes of the cells expressing them, they are soluble and therefore secreted into the supernatant media surrounding the cells. The Class I MHC molecules produced in this manner are collected as 'harvests' from a hollow fiber bioreactor sysem.

[0022] A 'construct' encoding a soluble Class I MHC molecule is produced by creating the truncated PCR product described above and placing it in a DNA vector that contains a promoter which is required for expression of the DNA. The construct is introduced into a mammalian cell line so that it can be

expressed; for example, a human B-lymphocyte line which is mutated so that it does not express any class I molecules other than the one coded for in the construct may be utilized, thereby easing purification of the desired Class I MHC molecules since no other Class I molecules are present. The advantages of this system are that (i) the resultant soluble Class I MHC molecules are produced and folded 'naturally' (since they are generated within mammalian cells, rather than made using either bacteria or insect cell lines; we furthermore have published data confirming that the soluble Class I MHC molecules appear to bear functional properties identical with those of full-length, cell surface-expressed HLA molecules), (ii) due to continuous secretion, large quantities of soluble Class I MHC molecules can be obtained with relative ease using hollow-fiber bioreactor systems, and (iii) the product yielded is significantly "cleaner" to begin with than the small quantities obtained by traditional cell lysate/immunoprecipitation protocols previously described. Edman sequencing and mass spectrometry show that the protein content of the material obtained from such harvests consists of soluble HLA molecules of a single, individual class I MHC molecule.

[0023] The total steps necessary to produce cell lines prior to growth in the hollow fiber bioreactor systems is considered a 'limiting factor' in that it requires multiple factors including (i) subcloning/sequencing to obtain specific constructs, (ii) transfection/screening by ELISA of mammalian cells to obtain

cell lines which have taken up the completed DNA vector for expressing the molecule, (iii) limiting dilution subcloning/screening by ELISA to obtain cell lines of maximum soluble HLA production levels, and (iv) RT-PCR/sequencing to validate cell lines before bioreactor culture.

[0024] The Class I MHC molecules produced by the method of the present invention overcome the disadvantages and defects of the prior art in that individual rather than multiple MHC molecules are present, and the individual MHC molecules produced by the method of the present invention more closely resemble human proteins than the individual MHC molecules currently produced in bacterial or insect cells. The individual MHC molecules produced in human cells by the method of the present invention may be utilized for HLA crossmatching, absorption/removal of anti-HLA antibodies from patients, development of HLA presented peptide based vaccines, discovery of pathogen based peptide epitopes for vaccine use, characterization of immune effector cells, and the like.

[0025] The method of the present invention produces individual MHC molecules in sufficient quantity for numerous experimental and clinical applications. The MHC molecules are produced from human cells and in all measurable ways resemble surface bound MHC molecules found on healthy human cells. Chaperone interaction, peptide loading, and antibody reactivity are all normal.

DETAILED DESCRIPTION OF THE INVENTION

[0027] The method of the present invention begins with the isolation of a mammalian DNA specimen or source containing multiple alleles or forms of the MHC. For example, an EBV (Epstein-Barr virus) transformed cell line may be utilized as it is readily commercially available. Alternatively, normal mammalian DNA may be utilized, or a virus transfomed cell line or an immortalised cell line may be constructed by known methods.

[0028] Total RNA is isolated from the source by known methods, such as by using Qiagen RNA extraction kit or similar separation means. The total RNA contains mRNA for the one or more MHC alleles. The mRNA is then reverse transcribed to form cDNA.

[0029] The allelic cDNA is then amplified by PCR using *Pfu* polymerase or other similar enzyme and flanking oligonucleotide primers. The primers are designed to amplify a segment of DNA that encodes a Class I MHC gene and truncates the Class I MHC gene by removal of the regions that encode the cytoplasmic and transmembrane domains of the Class I MHC molecule, thereby truncating the Class I MHC molecule encoded by the PCR product so that the normally membrane bound peptides will be secreted in the surrounding solution. The primers may also be designed to add a tail to the expressed protein to aid in purification thereof. For example, if it is desired for the secreted peptides to have histidine tails, a primer such as 3PEI-His is used. Tails

other than histidine tails should be equally useful. The truncated PCR product may then be purified using a Qiagen PCR purification kit or similar apparatus, and the PCR product sequenced to confirm the identity and fidelity of the PCR product.

[0030] The PCR product is then introduced into a mammalian expression plasmid or vector, such as, but not limited to, the pcDNA3.1 vector, by methods well known to a person having ordinary skill in the art, thereby creating the desired construct.

[0040] A suitable host cell, such as cell line 721.221 or an immortalised cell line that lacks expression of Class I MHC molecules is chosen and electroporated or transfected with the construct. The host cells containing the construct are isolated and screened to identify the most active HLA producing cells, and these cells are then used to inoculate media in roller bottles or similar containers where small amounts of individual MHC molecules are desired, or media in a hollow fiber bioreactor unit where large scale continuous production of individual MHC molecules are desired.

[0041] Described herein below is one Example of the method of the present invention. However, the steps outlined herein below are only provided for the purposes of example only and are not to be construed as limiting.

[0042] In step 1, a cell line containing multiple human MHC alleles was isolated. Total RNA was then extracted from the cell line using Qiagen RNA

extraction kit or similar separation means, and the mRNA present in the total RNA was reverse transcribed to form cDNA. This step is typically performed using a kit, such as a kit produced by Amersham-Pharmacia Biotech, and reverse transcriptase.

[0043] The cDNA encoding the desired MHC Class I allele was then amplified by PCR using *Pfu* polymerase or other similar enzyme and flanking oligonucleotide primers. The primers utilized in the PCR reaction were designed to remove the sequences encoding the cytoplasmic and transmembrane domains of the Class I MHC moleule, so that the resulting PCR product only encodes a secreted form of the Class I MHC molecule. In addition, the primer 3PEI-His was utilized so that the secreted form of the Class I MHC molecule encoded by the PCR product has a histidine tail attached thereto for aiding in purification of the secreted Class I MHC molecules. The truncated PCR product was then purified using a Qiagen PCR Purification kit or similar apparatus.

[0044] To clone the PCR product into the mammalian expression vector pcDNA3.1, the PCR product was cut with the restriction enzymes *EcoRI* and *XbaI* and ligated with the vector using T4 ligase. To ease in sequencing of the construct, the ligated PCR product and vector were transformed into *E. coli* JM-109 and plated on LB-ampicillin plates. The construct was extracted from antibiotic resistant colonies using a Promega Wizard mini prep kit, and the presence of the PCR product was confirmed by restriction enzyme digestion

using *CcoR* and *XbaI*. The PCR product was then sequenced using primers such as BGH, 3S and T7 and an Amersham Pharmacia Sequencing kit to confirm the identity and fidelity of the DNA sequence of the PCR product and to make certain the insert had no errors.

[0045] The plasmid containing the PCR product insert was then extracted from 45 mls of solution using a Qiagen Midi kit or other similar kit, and the plasmid or vector containing the PCR product was electroporated or transfected into 721.221 cells. G418 resistant cells were selected and screened for most active HLA producing cells by limiting dilution and ELISA assay. For example, the G418 resistant cells were serially diluted to a point where there was one cell per well, and an ELISA assay was used to determine which cells were producing the most soluble HLA.

[0048] The most prolific cells were grown in complete media in roller bottles or similar containers. A portion of the cells growing in the roller bottles were used to inoculate a hollow fiber bioreactor unit.

[0049] The inoculate from the roller bottles was fed counter current to harvest media in the hollow fiber bioreactor unit for continous large scale production of desired secreted Class I molecules. The inoculate was fed into the hollow fiber bioreactor unit at a rate to maintain optimum cell growth. Oxygen, glucose and carbon dioxide were fed into the temperature controlled circulating stream with feed rate, circulating rate, oxygen, glucose, carbon

dioxide and pH all controlled to provide a harvest rate that is maintained at a desired level of soluble Class I MHC, for example, a concentration of about 3μg/ml of secreted class I pure protein having antigen bound thereto may be obtained. The hollow fiber bioreactor units can be used for continuous, large scale production of large quantities of secreted individual Class I HLA molecules.